Immunofluorescence microscopy for the rapid diagnosis of melioidosis

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Abstract

A direct immunofluorescent antibody test (DIF) was developed for the rapid diagnosis of melioidosis, a potentially fatal infection caused by *Pseudomonas pseudomallei*. In a clinical evaluation of 369 sputum, pus, or urine specimens from 272 patients with suspected melioidosis, the DIF had a sensitivity of 73% and a specificity of 99% compared with culture. Using this DIF, a confident diagnosis of melioidosis can now be made within two hours of admission to hospital, compared with the delay of two to four days required for culture results. Consequent early institution of specific antimicrobial therapy may help to save lives.

(J Clin Pathol 1994;47:377–379)

Pseudomonas pseudomallei is an important human and animal pathogen in Southeast Asia and northern Australia.[1 2] In Thailand, most human melioidosis is seen in the large rice farming community in the northeastern region of the country, where it accounts for 20% of community acquired septicaemias.[3] The spectrum of disease ranges from life threatening severe sepsis to mild localised infections. The clinical manifestations of melioidosis are protean, making a diagnosis difficult on clinical grounds alone. The mortality of severe disease remains high, despite recent improvements in treatment.[4 5] *Ps pseudomallei* is intrinsically resistant to the commonly used empirical treatment for severe sepsis in Thailand, a penicillin plus gentamicin, and culture techniques require at least 48 hours to identify *P pseudomallei* in clinical specimens.[6] Delays in diagnosis could prove fatal, and therefore the use of rapid diagnostic methods is important. A direct immunofluorescent antibody test (DIF) for the rapid detection of *Ps pseudomallei* in clinical samples has been developed.

Methods

CONJUGATE PREPARATION

Rabbits were immunised with a heat killed suspension (10^9 colony forming units/ml) of *Ps pseudomallei* (strain 204, a clinical isolate from Ubon Ratchatani, Thailand) given intravenously in increasing doses every three to four days. Serum was collected three days after the fifth dose and fractionated (by protein A-sepharose CL-4B chromatography) to obtain purified polyclonal anti-*Ps pseudomallei* immunoglobulin G (IgG). Using the method of Samuel *et al*,[7] the antibody was then conjugated to fluorescein isothiocyanate (FITC). In summary, the IgG was diluted in carbonate buffer at pH 9·2, FITC (5 mg/ml) was added in a 20:1 molar ratio and the mixture was left in the dark at room temperature for one hour. Unbound FITC was removed by filtration through Sephadex G25. Conjugate fractions were pooled and stored at 4°C in phosphate buffered saline (PBS) containing 33% glycerol and 0·02% thiomersal.

DIF METHOD

Air dried smears were fixed in methanol for 30 seconds. The slides were flooded with blocking buffer (PBS with 3% bovine serum albumin and 3% normal rabbit serum), incubated at room temperature for 30 minutes and rinsed with PBS. They were then flooded with a 1:400 dilution (in blocking buffer) of the conjugate, incubated at room temperature for 1 hour in the dark, and finally washed in PBS for 15 minutes before drying on a hotplate. After mounting in buffered glycerol (pH 9), the slides were examined by incident light fluorescence, using a $\times 100$ oil immersion lens. A positive result was observed when the periphery of the bacilli showed strong apple-green fluorescence. A known positive control slide was included in each batch of tests.

LABORATORY EVALUATION

Fifty-one isolates of *Ps pseudomallei* (from patients in northeastern Thailand), 22 strains of other pseudomonas species and 22 different Enterobacteriaceae were tested. All strains were identified by API 20NE or API 20E (BioMerieux UK Ltd, Basingstoke, UK), and ancillary tests as described previously.[8] Suspensions of organisms were made in PBS, to a density of 10^5/ml. Aliquots of 5 μl were then spotted onto teflon coated multiwell slides and air dried.

CLINICAL EVALUATION

Samples of sputum, pus, wound swabs, and urine were obtained from 272 patients with suspected melioidosis in Sappasitprasong Hospital, Ubon Ratchatani, Thailand.[3] Two thin smears were made from each sample, one for Gram staining and the other for DIF. All specimens were inoculated onto selective and non-selective agar, and into a selective broth as described previously;[6] pus and other

This work was presented in part at the XIIIth International Congress of Tropical Medicine and Malaria, Pattaya, Thailand, November 29–December 4, 1992. Abstract number THP11-4.

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Accepted for publication 14 October 1993

body fluids were also put into tryptic soy broth. All solid media were incubated for four days to allow for typical colonial morphology to develop. Enrichment broths were subcultured after 48 hours' incubation at 42°C. Suspect colonies of *Ps pseudomallei* were identified by standard methods[8] and latex agglutination.[9]

Results

In the initial evaluation using cultures of fully identified organisms, the DIF was 100% specific for *Ps pseudomallei*. All 51 strains of *Ps pseudomallei* exhibited positive fluorescence, while none of the other *Pseudomonas* spp. (including those strains of *Ps cepacia* which agglutinate non-specifically in the *Ps pseudomallei* latex test)[9] or Enterobacteriaceae gave a positive result (table 1).

In the clinical study, *Ps pseudomallei* was isolated from 150 of 369 clinical specimens. One hundred and twelve were positive by DIF (table 2); two of these were false positives and 110 were true positives, giving an overall sensitivity of 73% compared with culture, with a specificity of 99%. The false positives both occurred in sputum specimens: one subsequently grew *Ps aeruginosa* and the other *Ps cepacia*. Broth cultures of each organism were retested by DIF, but were consistently negative. *Ps pseudomallei* could not be isolated from any specimen from either patient. The positive and negative predictive values of DIF were 98% and 85%, respectively. Gram negative rods were seen in 203 (55%) specimens: 105 were negative by DIF and culture, two gave false positive fluorescence, and four specimens were DIF negative but subsequently grew *Ps pseudomallei*. Of these four, three specimens had a heavy growth of coliforms, while the fourth was cultured on selective media alone, so the presence of other organisms could not be determined. Of the 92 DIF and culture positive specimens, only 41 (45%) contained Gram negative rods which were described as "typical" or bipolar. Overall, Gram stain alone had a diagnostic sensitivity of 62% and a specificity of only 50%. Gram negative rods were not seen in 18 of 110 (16%) specimens truly positive by DIF. There were 40 (27%) false negative DIF results. Of these, 16 specimens gave only light growth of *Ps pseudomallei* (<20 colonies) on direct plating, 10 grew <5 colonies, and the remaining 14 were cultured only from selective enrichment broth.

Table 1 Immunofluorescence results for positive and negative control organisms

Organism	*Number tested*	*Number positive*
Ps pseudomallei	51	51
Pseudomonas species		
Ps cepacia	8	0
Ps aeruginosa	3	0
Ps stutzeri	3	0
Others	8	0
Enterobacteriaceae		
Klebsiella spp.	6	0
Escherichia spp.	3	0
Enterobacter spp.	3	0
Citrobacter spp.	3	0
Proteus spp.	2	0
Others	5	0

Table 2 Immunofluorescence results for 369 clinical specimens

Specimen type (no. tested)	*GNR seen*	*DIF positive*	*Culture positive*	*Sensitivity of DIF (%)*
Sputum (155)	117	52*	62	81
Wound swabs (68)	20	21	38	55
Pus or fluid (90)	35	31	41	76
Urine (56)	31	8	9	89
Overall (369)	203	112	150	73

GNR = Gram negative rod
* Includes two false positives

Discussion

The majority of patients with melioidosis are not diagnosed until a positive culture result is obtained, even in endemic areas where the index of suspicion is high. Inappropriate treatment due to delayed diagnosis contributes to the high mortality of this disease.[3] In films of pus or sputum, melioidosis is often diagnosed if bipolar staining ("safety-pin") Gram negative rods are seen,[1] but this finding is not specific. In this study only 41/150 (27%) of specimens containing *Ps pseudomallei* had "typical" Gram negative rods on direct microscopy, and similar "safety-pin" bacilli were also seen in 9% of smears from specimens which grew other Gram negative organisms.

Culture remains the gold standard for the definitive diagnosis of melioidosis. Isolation of *Ps pseudomallei* from blood or purulent exudates is not difficult, but problems may be encountered with specimens likely to have a heavy contaminating flora, such as sputum and throat swabs. The relatively slow development of typical colonies, even on non-selective media, makes recognition of *Ps pseudomallei* amongst mixed growth difficult.[5] If growth occurs only after enrichment, then the delay to positive culture will be prolonged from two to four days.

The DIF method was originally developed for use on environmental specimens and exudates from experimentally infected animals.[10] Recently a DIF method was evaluated in a small number of cases of suspected human melioidosis (Naigowit P, *et al.* Abstract presented at conference on medical laboratory technology, 1991). Sensitivity was 83% but positive fluorescence occurred in 6/44 samples which did not grow *Ps pseudomallei* (although selective enrichment broths may not have been used); the specificity was therefore only 88%. In the present study, specificity was 99% and sensitivity was 73%. The negative predictive value for a specimen in which Gram negative rods were seen but the DIF was negative was 96%. Cultures from the false DIF negatives indicated that 60% contained a very scanty number of organisms, 35% growing from enrichment broth only. It is estimated that 10^4–10^5 organisms/ml are required for detection by light or fluorescent microscopy. The two apparently false positives are more difficult to explain. False positive organisms were not encountered during

the laboratory evaluation, but it should be noted that neither *Pseudomonas mallei* nor *Legionella pneumophila*, both of which are known to cause serological cross-reactions with *Ps pseudomallei*, were available for testing. It is possible that small numbers of *Ps pseudomallei* may have been overgrown by the other Gram negative rods which were present in the specimens. One patient had received prior treatment with chloramphenicol, and it is also possible that *Ps pseudomallei* was present but failed to grow in the presence of the antibiotic.

It is now possible to have a specific result within two hours in three out of four patients with melioidosis, if appropriate specimens are available (in this study, about 85% of patients were found to have specimens suitable for DIF on admission). For patients with no focal lesions, detection of urinary antigen may provide a rapid diagnosis. Reagent costs for the DIF are low: approximately £50 for 1 ml of conjugate (400 tests) and £3 for 100 ml of blocking buffer. Unfortunately, the technique requires an expensive fluorescence microscope. However, the recent development of a simple filter which modifies a standard light microscope for fluorescent work[11] may allow peripheral laboratories in endemic areas to use this methodology. This method may also be adaptable to non-fluorescent visualisation, i.e. using horseradish peroxidase labelled antibodies and an appropriate chromogenic substrate. These strategies are currently being investigated.

The authors wish to thank Mr Sayan Langla for technical assistance; Dr T L Pitt and Khun Pimjai Naigowit for expert advice and encouragement; and the Director, medical, laboratory and nursing staff of Sappasitprasong Hospital for their continuing support. This study was part of the Wellcome-Mahidol University, Oxford Tropical Medicine Research Programme, funded by the Wellcome Trust of Great Britain.

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